

AMRL-TR-79-43

ADA #74631

reduction



METABOLISM OF HYDRAZINE

F. N. DOST

D. L. SPRINGER

B. M. KRIVAK

DEPARTMENT OF AGRICULTURAL CHEMISTRY

D. J. REED

DEPARTMENT OF BIOPHYSICS AND BIOCHEMISTRY

OREGON STATE UNIVERSITY

CORVALLIS, OREGON 97331

AUGUST 1979

20060706054

Approved for public release; distribution unlimited.

AEROSPACE MEDICAL RESEARCH LABORATORY
AEROSPACE MEDICAL DIVISION
AIR FORCE SYSTEMS COMMAND
WRIGHT-PATTERSON AIR FORCE BASE, OHIO 45433

STINFO COPY

NOTICES

When US Government drawings, specifications, or other data are used for any purpose other than a definitely related Government procurement operation, the Government thereby incurs no responsibility nor any obligation whatsoever, and the fact that the Government may have formulated, furnished, or in any way supplied the said drawings, specifications, or other data, is not to be regarded by implication or otherwise, as in any manner licensing the holder or any other person or corporation, or conveying any rights or permission to manufacture, use, or sell any patented invention that may in any way be related thereto.

Please do not request copies of this report from Aerospace Medical Research Laboratory. Additional copies may be purchased from:

National Technical Information Service
5285 Port Royal Road
Springfield, Virginia 22161

Federal Government agencies and their contractors registered with Defense Documentation Center should direct requests for copies of this report to:

Defense Documentation Center
Cameron Station
Alexandria, Virginia 22314

TECHNICAL REVIEW AND APPROVAL

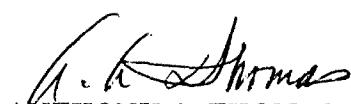
AMRL-TR-79-43

The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Information Office (OI) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER



ANTHONY A. THOMAS, MD

Director
Toxic Hazards Division
Aerospace Medical Research Laboratory

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER AMRL-TR-79-43	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) METABOLISM OF HYDRAZINE		5. TYPE OF REPORT & PERIOD COVERED Final 17 Jan 77 - 17 Mar 79
		6. PERFORMING-ORG. REPORT NUMBER
7. AUTHOR(s) F. N. Dost, D. L. Springer, B. M. Krivak and D. J. Reed		8. CONTRACT OR GRANT NUMBER(s) F33615-77-C-500
9. PERFORMING ORGANIZATION NAME AND ADDRESS Department of Agricultural Chemistry Oregon State University Corvallis, Oregon 97331		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61101F, 2312-V1-116
11. CONTROLLING OFFICE NAME AND ADDRESS Aerospace Medical Research Laboratory Aerospace Medical Division Air Force Systems Command Wright-Patterson Air Force Base, Ohio 45433		12. Report Date AUGUST 1979
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		13. NUMBER OF PAGES 23
		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
16. DISTRIBUTION STATEMENT (of this Report) Approved For Public Release: Distribution Unlimited		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)		
18. SUPPLEMENTARY NOTES		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) Nitrogen-15 Hydrazine Metabolism Respiratory Gases		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) A method for measuring $^{15}\text{N}_2$ expired by rats following administration of ^{15}N-labeled substrates has been devised. Measurement sensitivity was below 10 $\mu\text{moles } ^{15}\text{N}_2$ produced over a 24 hour period. Studies of the metabolic disposition of ^{15}N-hydrazine indicated that over a 48 hour period about 25% of a single, 1 mmole/kg dose was converted to $^{15}\text{N}_2$. Urinary hydrazine accounted for almost 29% of the dose over 48 hours, and a hydrolyzable derivative(s) of hydrazine accounted for about 24%; total respiratory and		

urinary excretion accounted for about 75% of the dose. In the blood, both components were measureable for at least 24 hours. The derivative is thought to be mono- or diacetylhydrazine but identification has not been clearly established. Measurements during continuous infusion of hydrazine showed that at dose rates below 0.167 mmole/kg/hour, blood hydrazine usually reached a steady state proportional to input rate.

SUMMARY

A method for measuring $^{15}\text{N}_2$ expired by rats following administration of ^{15}N -labeled substrates has been devised. Measurement sensitivity was below 10 μmoles $^{15}\text{N}_2$ produced over a 24 hour period. Studies of the metabolic disposition of ^{15}N -hydrazine indicated that over a 48 hour period about 25% of a single, 1 mmole/kg dose was converted to $^{15}\text{N}_2$. Urinary hydrazine accounted for almost 29% of the dose over 48 hours, and a hydrolyzable derivative(s) of hydrazine accounted for about 24%; total respiratory and urinary excretion accounted for about 75% of the dose. In the blood, both components were measureable for at least 24 hours. The derivative is thought to be mono- or diacetylhydrazine but identification has not been clearly established. Measurements during continuous infusion of hydrazine showed that at dose rates below 0.167 mmole/kg/hour, blood hydrazine usually reached a steady state proportional to input rate.

PREFACE

The research described in this report was conducted in the Department of Agricultural Chemistry, Oregon State University, Corvallis, Oregon 97331, under Contract F33615-77-C-0500, Project 2312, Task 2312V1, Work Unit 2312V116. The principal investigator was Dr. F. N. Dost. Contract monitor for 6570th Aerospace Medical Research Laboratory was Dr. K. C. Back, Chief, Toxicology Branch, Toxic Hazards Division.

INTRODUCTION

The extensive use of hydrazine in both military and industrial applications has necessitated a thorough toxicologic and hazard evaluation. In spite of a great deal of study over recent decades, there are still serious gaps in understanding the biological effects of hydrazine. Similarly, the metabolic fate of hydrazine is not well defined.

This report describes research which further defines the behavior of hydrazine once it enters the animal. The existing literature in this area of research is limited. One reason is that isolation and identification of simple nitrogen compounds is often difficult since no radioisotope of nitrogen is available, and use of tritium labeling is futile in view of extensive exchange reactions. There has also been frustration over the apparent "disappearance" of hydrazine after administration; no one has succeeded in establishing a complete general inventory.

The impetus to learn the details of reactions of hydrazine with biological entities has been intensified recently with the possibility that hydrazine may be a weak carcinogen. The presence of small amounts of hydrazine in tobacco smoke (derived from synthetic plant growth regulators) adds to the importance of the problem (Liu et al., 1974).

Metabolic study of hydrazine as a toxic substance began only in the early 1950s. McKennis et al., (1955) found that a variable but substantial amount of hydrazine emerged in urine of dogs within 48 hours after acute dosage with 15 mg/kg hydrazine, a treatment which is frequently lethal.

Dambrauskas and Cornish (1964) followed the time course of tissue levels and excretion of hydrazine as measured spectrophotometrically following reaction with p-dimethylaminobenzaldehyde. Hydrazine continued to appear in urine for 20 hours or more after a single injection, and tissue concentrations decreased steadily over the same period. About 50% of the administered hydrazine could not be found in either tissues or urine and was assumed to have been metabolized. The amount "metabolized" was almost constant throughout the time course, indicating that it was either lost experimentally or was not subject to the processes mediating removal of presumably intact hydrazine from tissues.

Very few *in vivo* chemical reactions of hydrazine have been documented. McKennis et al. (1959) observed significant diacetylation of hydrazine in rabbits but found that such conversion in the dog was limited. Acetylation of many hydrazine derivatives, notably isoniazid, is known to be genetically determined within humans (Evans et al., 1960; Dufour et al., 1964) and possibly other species, and the significance to other hydrazine reactions of such differences in this pathway can only be speculated upon.

Hydrazine is a carbonyl reagent and is generally accepted as being reactive *in vivo* with pyridoxal phosphate and possibly other aldehydes and ketones. Elevation of blood ammonia by hydrazine (McKennis and Weatherby, 1956) suggests that some hydrazines can cause alteration in ammonia metabolism or even may proceed to the formation of ammonia. Hydrazine was used in studying

the physiology and chemistry of hemoglobin around the turn of the century; the reaction was stated by Buckmaster (1913, 1914) to form gaseous nitrogen.

In the work reported here, we have devised a method for measuring nitrogen gas derived from hydrazine, using stable isotope methodology. A number of studies of hydrazine excretion rates and behavior in the circulation have also been made using conventional chemical analytical methods. We have found that about 15% of a single dose of ^{15}N -hydrazine was converted to nitrogen-15 gas within 30 minutes after administration, and that an additional 10% emerged in the subsequent 24-48 hours. The extent of conversion was not significantly influenced by dosage. Measurable hydrazine remained in blood at least 24 hours after a single injection, as did a hydrolyzable hydrazine metabolite. Urinary excretion continued for at least 48 hours to the extent of about 50% of dose. Hydrazine infusion at low dose rates resulted in a constant blood hydrazine concentration for the duration of infusion.

METHODS

Measurement of $^{15}\text{N}_2$ in respiratory gases

The problem to be solved in measuring volatile nitrogen-15 in respiratory gases of rats can be summarized briefly: Very small amounts of a non-condensable gas must be quantitatively recovered from a closed atmosphere in a system that can support life in an intact animal for an indefinite period. Several requirements are obvious. The system should: 1) utilize a filling gas that is condensable or otherwise separable, and non-toxic; 2) be gas-tight at ambient pressure and able to withstand substantial vacuum; 3) be designed to permit acute or continuous injection of intoxicants or other agents to experimental animals without opening the system; 4) have a sensitive, demand-driven oxygen supply; and 5) include CO_2 and water traps that do not otherwise compromise the experimental atmosphere. At the end of a metabolism period there must be associated capability to remove oxygen, remove the bulk condensable gas, and trap $^{15}\text{N}_2$ or other noncondensable gases and move them to a mass spectrometer for analysis.

Construction of the System: The system described includes an animal chamber, soda lime and Drierite traps for CO_2 and water, a circulatory pump, an O_2 replenishing system, and a source of sulfur hexafluoride, the bulk gas of the system (Figure 1). The components were designed and fabricated locally, and assembled with "O" ring joints. Soda lime and Drierite traps were made of 2 inch Pyrex pipe, with "O" ring joints, which were clamped with bolted flanges to assure a gas-tight seal. Demountable components were joined by hand-tightened Ultratorr fittings (Cajon Company) which seal to more than 10^{-4} Torr. Stainless steel flexible joints (Cajon Company) were used where flexibility was necessary.

The animal chamber was constructed in two pieces from 3-1/2" Corning Pyrex sewer pipe, using the standard joint and clamp, fitted with a Teflon gasket for vacuum seal. A removable urine trap and a port for insertion of a watering tube were provided. Indwelling cannulas were attached to 22 g needle tubing inserted through a soft rubber stopper in a port at the top of the chamber.

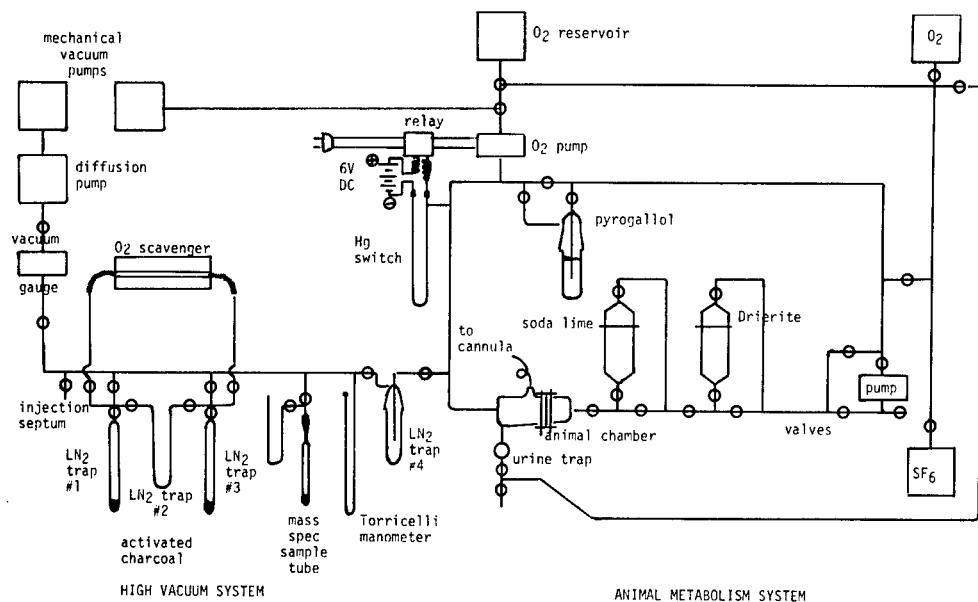


Figure 1. Schematic of apparatus for collection and separation of noncondensable gases in respiration of rats.

The O_2 replenishing system was controlled by a mercury manometer pressure switch, which in turn activated a relay to the peristaltic oxygen pump. The pump drew directly from the oxygen tank through a low pressure regulator and served as a regulator of oxygen flow into the system. A record of pumping time can serve as an index of oxygen consumption if desired. Ultrapure oxygen (99.995% O_2 , Airco Products) was used in all phases of this study to avoid buildup of argon, which is noncondensable and may accumulate from less pure oxygen in sufficient volume to interfere with collection and mass spectrometric determination of $^{15}N_2$ at the end of the experimental period.

The vacuum system was modified extensively from a Delmar portable high vacuum system (Delmar Scientific Laboratories, Inc.). The primary manifold was constructed according to Figure 1 from 1" glass tubing with vacuum valves from Kontes Glass or J.T. Young.

Mass spectrometer sample tubes were constructed according to Figure 2 and were loaded with 0.5 g charcoal. This construction enabled sample storage for several days if necessary and allowed easy transport to the mass spectrometer.

Preparation and Start-Up of an Experiment: Each of the components of the system must be prepared in the proper sequence to prevent inward leakage of atmospheric gases. Before each experiment the charcoal traps were heated for 5 minutes with an external 250°C airstream while being evacuated to remove any gases remaining on the charcoal from previous experiments. This step is particularly important when Freon-14 is used as an internal standard. The

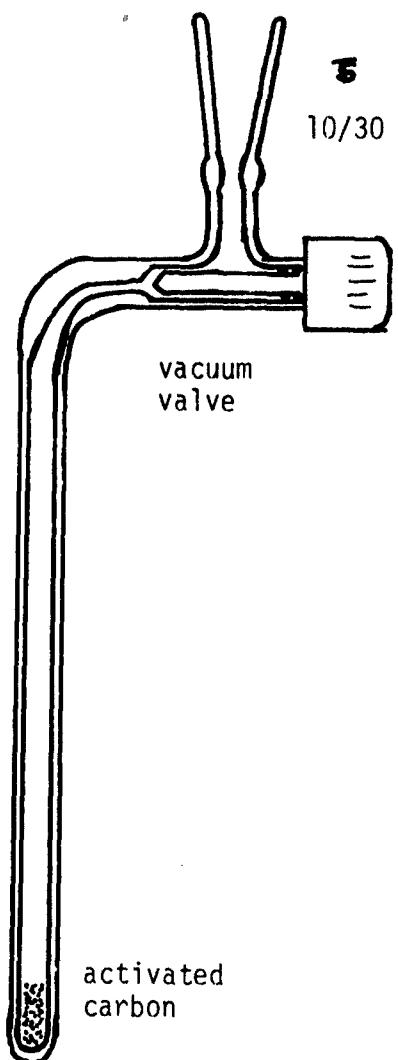


Figure 2.
Mass spectrometer sample tube.

pyrogallol (PGA) trap to be used for oxygen scavenging at the end of the experiment was filled with 200 ml of aqueous 50% KOH containing 30 g of pyrogallic acid, flushed for 10 minutes with SF₆ (1.0 liter/min), and attached according to Figure 1. The animal was then placed in the restrainer and inserted into the animal chamber which was then flushed for 15 minutes with 100% O₂, with care to avoid excessive pressure changes. The remaining portion of the system was then isolated, evacuated, and refilled with O₂. With the circulating pump operating, the entire animal system was flushed for 10 minutes with 100% O₂, then with a 1:4 mixture of O₂-SF₆ for an additional 15 minutes at a flow rate of 1.25 liter/min. The system was then closed, the oxygen replenishment pump switched on, and the chamber atmosphere circulated continuously throughout the duration of the experiment. Single experiments have ranged from 0.5-24 hours. At the end of each experiment the animal was euthanized, usually by injection of pentobarbital through an indwelling cannula.

Removal and Separation of Gas Mixtures: After euthanizing the animal, the atmosphere was cycled through the pyrogallol trap for 15 minutes, which removed about 90% of the O₂. Gases were then moved from the animal system

into the vacuum manifold by cooling trap #1 (Fig. 1) containing 1.0 g of activated carbon. (The adsorptive capacity at low temperatures is sufficient to generate negative pressures of approximately 10^{-3} torr in the vacuum manifold.) Traps 2 and 4 were also cooled with liquid nitrogen and as the gas mixture passed through, most of the SF₆ was removed. Any remaining SF₆ was removed by one or more additional passes across trap 2, by alternately cooling and warming traps 1 and 3.

The remaining O₂ was removed with an Oxy-absorbent (Altech Associates) column heated at 200°C in a stainless steel column. One pass was sufficient to effectively remove the remaining O₂ without loss of ¹⁵N₂. This transfer was also powered by temperature shifting with liquid N₂ from trap 1 to trap 3. The Oxy-absorbent column can be recharged with hydrogen gas at 135°C for 2 hrs.

The noncondensable gases were moved onto the charcoal of the mass spectrometer sampling tube at liquid N₂ temperature and the volume estimated manometrically. In a typical experiment, the gas in the sample tube included 0.2 - 1.0 ml ¹⁵N₂ at standard temperature and pressure (STP) from administered ¹⁵N-hydrazine sulfate, varying amounts of ¹⁴N₂ and O₂ leaked from the atmosphere, small amounts of CH₄ from intestinal bacterial metabolism, and argon as an O₂ impurity, with a total volume of 20-40 ml. If needed, ¹⁴N₂ was added to bring the sample to a constant volume approximately equivalent to the volume of the standard, the valve was closed and the device detached for transport to the mass spectrometer. The maximum volume that can be accepted by the mass spectrometer without loss of response linearity is about 40 ml. Volume of undesirable gases can be decreased in longer experiments by blanketing the entire system with a large polyethylene glove bag inflated with CO₂, which is automatically scavenged by the traps in the circuit if it leaks in. This approach is necessary only during experiments longer than 24 hours.

Mass Spectrometer Quantitation: The mass spectrometer used in this program was a Varian CH-7 equipped with a viscous flow inlet. Entry into the sample cavity was slow enough that an insignificant amount of sample gas was lost over a 5 minute measuring cycle.

There is an inherent variability in the output of the mass spectrometer which is designed primarily for compound identification and not quantitation. The system also varies in response with sample volume. Under these circumstances, the most convenient method for mass measurement was based on an internal standard, or "spike", of known volume introduced into the vacuum manifold to mix with the ¹⁵N₂ sample. Freon-14 (CF₄) is most satisfactory, although other gases could probably also be used. The major masses observed for Freon-14 are m/e 69 (CF₃⁺) and m/e 50.

To establish the validity of the methods a standard curve was prepared with constant volumes of Freon-14 (1.0 ml) and volumes of ¹⁵N₂ between 0.25 ml and 2.5 ml. The total sample volume was adjusted with ¹⁴N₂ to 25 ml in each case. The ratio of mass spectrometer output for m/e 30 to m/e 69 plotted against the volume of ¹⁵N₂ was linear for a sample volume range from 5-40 ml. All values were corrected to standard temperature and pressure.

Recovery of $^{15}\text{N}_2$ from the animal system was tested by complete oxidation of ^{15}N -hydrazine sulfate to $^{15}\text{N}_2$ in the presence of Cu^+ and H_2O_2 (Wellman et al., 1976). Similarly ^{15}N -ammonium chloride was quantitatively converted to $^{15}\text{N}_2$ by the method of Ross and Martin (1970).

Capability for recovery of $^{15}\text{N}_2$ from the entire animal system was evaluated by injecting a known volume of $^{15}\text{N}_2$ through a serum cap into the animal system containing a typical test atmosphere, circulating for 2 hours, and collecting and transferring as described. The amount of $^{15}\text{N}_2$ recovered was 101.3% with a standard deviation of 5.75% (Table 1). Similarly, to determine the recovery from an animal, $^{15}\text{N}_2$ was infused very slowly through a cannula in the posterior vena cava of a rat and collected. Recovery was 89.1 ± 5.38 (Table 1). All recoveries from hydrazine treated animals were corrected according to that factor. $^{15}\text{N}_2$ injected intraperitoneally was absorbed very slowly, resulting in low recovery, even on relatively long experiments.

TABLE 1
COMPARISON OF RECOVERY OF $^{15}\text{N}_2$ GAS ADMITTED INTO
THE EMPTY ANIMAL SYSTEM WITH RECOVERY OF GAS
INFUSED INTRAVENOUSLY INTO THE RAT.

<u>Administration of $^{15}\text{N}_2$ gas</u>	<u>Number of Samples</u>	<u>Recovery of $^{15}\text{N}_2$ (%)</u>	<u>Coefficient of Variation (s/\bar{x})</u>
Empty chamber	10	101.3 ± 5.75^a	0.0565
Intravenous	10	89.1 ± 5.38^a	0.604

^aPercent of the total injected \pm the standard deviation of the mean.

Recovery of $^{15}\text{N}_2$ after chemical oxidation of ^{15}N -hydrazine sulfate was $101 \pm 7.66\%$; recovery from oxidized ^{15}N -ammonium chloride was 94.4% (Table 2).

TABLE 2
IN VITRO OXIDATION OF ^{15}N -HYDRAZINE SULFATE OR ^{15}N -AMMONIUM CHLORIDE AND RECOVERY OF $^{15}\text{N}_2$ COMPARED WITH $^{15}\text{N}_2$ STANDARD GAS.

$^{15}\text{N}_2$ Standard Gas			
	<u>Number of Samples</u>	<u>Recovery of $^{15}\text{N}_2$ (%)</u>	<u>Coefficient of variation (s/\bar{x})</u>
$^{15}\text{N}_2$ standard gas	5	100 ± 3.65	0.036
^{15}N -hydrazine sulfate oxidation	5	101 ± 7.66^a	0.076
^{15}N -ammonium chloride	4	94.4 ± 9.24^a	0.098

^aPercent of the expected $^{15}\text{N}_2$ \pm the standard deviation of the mean.

$^{15}\text{N}_2$ and Freon-14 standards were transferred using 1.0 ml disposable plastic syringes, using 24 gauge, 3 1/2 inch needles to reduce exchange diffusion. The syringe reproducibility for gas transfer was tested using $^{14}\text{CO}_2$ generated in a closed system. One milliliter volumes of $^{14}\text{CO}_2$ were transferred in the syringe to an ethanol-ethanolamine trapping solution and later counted in a Beckman liquid scintillation counter. The coefficient of variation for 10 samples was 2.1%. Syringes were further standardized by weight, using water.

The sensitivity of the method was determined ultimately by the capability of the mass spectrometer. Our system was routinely employed to analyze from 0.02 to 0.1 mmole of $^{15}\text{N}_2$. Sensitivity below 0.02 mmoles is possible with preparation of appropriate standards. Equal volumes of $^{15}\text{N}_2$ and Freon-14 must be maintained to avoid scale expansion on the mass spectrometer during a given run. The variability within and among standards is shown in Table 3.

TABLE 3
VARIABILITY OF MASS SPECTROMETER
DETERMINATION WITHIN AND AMONG STANDARDS.

	m/e		
	30	69	30/69
10 scans from a single sample	0.019	0.012	0.018
11 standards prepared independently ^a	0.067	0.043	0.068

^aThe routine procedure was to average ten mass spectrometer scans for each determination.

Total sample volume was determined primarily by the amount of atmospheric $^{14}\text{N}_2$ which entered the system during the metabolism period. This contamination was partially time dependent, suggesting a consistent leak rate across the necessarily large number of joints. Sample size was arbitrarily set to exceed the leak rate for the period of the experiments, and both standards and unknowns were prepared in the same volumes.

Sample turnover time was dependent upon the rate of metabolism of the administered compound and the duration of the experiment. Set-up and collection for $^{15}\text{N}_2$ measurement required about 1.5 hours each; two short term animal experiments could be conducted in a 10 hour day. Accumulation of samples for one week allowed for efficient utilization of mass spectrometer time and preparation of the optimum number of standards.

Determination of ^{15}N in urinary ammonia.

A 24 hr urine sample was collected at dry ice temperature in acid from a rat that had been treated with ^{15}N -hydrazine sulfate (1.0 mmole/kg). Ammonia from

the entire sample was collected into a 16 mm by 50 mm shell vial containing 1.0 ml of 2.0 N HCl, according to the method of Conway (1957). After evaporation to dryness at 95°C, the crystalline ammonium chloride salt was converted to nitrogen gas by the method of Ross and Martin (1970) and Porter and O'Deen (1977). This method involves oxidation of ammonium chloride and collection of the nitrogen gas in mass spectrometry tubes.

For standardization, known amounts of $^{15}\text{NH}_4\text{Cl}$ were converted directly to nitrogen gas or added to urine from untreated rats, then treated as above. The amount of nitrogen-15 in the standards or spiked sample was determined by mass spectrometry as described above. Recovery from spiked control urine was consistently about 50% of the amount converted directly from $^{15}\text{NH}_4\text{Cl}$. Because samples from rats treated with ^{15}N -hydrazine contained no detectable ^{15}N in ammonia, further improvement of recoveries was not attempted.

Measurement of Hydrazine and Hydrazine Derivatives in Biological Fluids.

Hydrazine was analyzed in samples of urine and blood by a micromodification of the method of Reynolds and Thomas (1965), employing p-dimethylaminobenzaldehyde (DMBA) as the color reagent. Whole blood was diluted with enough water to rupture all erythrocytes, the lysate was treated with 10% trichloroacetic acid (TCA) to precipitate all protein, and then centrifuged. Samples were held on ice during each step prior to acidification. DMBA was added to an aliquot of the TCA extract, which was incubated 20 minutes at room temperature. Absorbance was read at 470 nm. The color was stable for at least two hours.

For determination of the hydrolyzable derivative(s) an aliquot of the TCA extract was acidified to 1N with HCl, incubated 48 hours at 45° and analyzed as above. Standards of hydrazine and diacetylhydrazine were prepared in rat serum for the respective assays. (Absolute identity of the hydrolyzable derivative(s) is not established but diacetylhydrazine is an appropriate model substrate.) The assay is linear between 0.006 and 0.09 μ moles in a final volume of 3 ml. Standards of each compound were usually prepared in the range between 0.01 - 0.06 μ moles.

Urine samples were diluted to volume with water, but since protein precipitation is not needed, HCl was used alone to acidify samples. There are several interfering substances in urine which react with the color reagent and which are variable from sample to sample. To correct for this interference, the hydrazine in the sample was oxidized with alkaline CuSO_4 (18 mM in 1N NaOH), and the sample reanalyzed to determine the background color. Standards were prepared by spiking unknown duplicate samples with hydrazine and diacetylhydrazine.

Animal Preparation, Sampling and Administration of Chemicals.

Male Sprague-Dawley rats were used in all phases of the work. All of the foregoing surgical procedures were performed under sodium pentobarbital anesthesia supplemented by ether on animals weighing 250-280 grams. At least four full days were allowed for recovery before use. Animals were surgically implanted with intraperitoneal or subcutaneous cannulas for administration of hydrazine. In some experiments, subcutaneous hydrazine was infused through a hypodermic

needle taped in place. As needed, cannulas for infusion, feeding, or sampling were also placed in the posterior vena cava and/or the small intestine. Bladder cannulas were installed under ether anesthesia the day before treatment.

Subcutaneous injection of chemicals leads to distribution that tends to parallel that of agents taken in by either inhalation or transcutaneous absorption. However, recoveries of $^{15}\text{N}_2$ from subcutaneous ^{15}N -hydrazine were comparatively low in initial experiments. While use of direct intravenous injection resulted in greater conversion, the variability was unacceptable. Intraperitoneal administration was found to be adequately consistent and was selected for the major series of intact animal studies with ^{15}N -hydrazine.

In the $^{15}\text{N}_2$ recovery experiments, the animal was placed in a restraining device, which was then placed in the metabolism chamber, the necessary cannulas were connected, and hydrazine or $^{15}\text{N}_2$ was administered as a single dose or as a continuous infusion.

During observation of blood hydrazine concentration and urinary excretion time courses, animals were held in acrylic restrainers for the duration of the experiment. Blood samples were drawn from the intravenous cannula in volumes of no more than 0.5 ml at one time, nor more than 2.25 ml in 24 hours. Urine was collected either directly on dry ice or into HCl at 4°. Fluid intake in long duration experiments was maintained by infusion of water into the small intestine.

Chemicals

Sulfur hexafluoride, 99.8%, and carbon tetrafluoride (Freon-14), 99%, Matheson Gas Products, East Rutherford, NJ; hydrazine sulfate ^{15}N (99 atom %) and ^{15}N -nitrogen, 99 atom %, U.S. Services, Inc., Summit, NJ; oxygen (ultra-pure), Liquid Air Inc., San Francisco, CA; oxy-absorbent, Altech; Drierite, W.A. Hammond Drierite Co., Xenia, Ohio; Sodasorb (soda lime), Dewey & Almy, Cambridge, MA; activated carbon, type Cal 12 x 40, Calgon, Pittsburgh, PA; pyrogallic acid, Mallinckrodt; p-dimethylaminobenzaldehyde (DMBA) and hydrazine, 99%, Eastman. Diacetylhydrazine was synthesized by the method of Turner (1947). All other chemicals were reagent grade.

RESULTS

Production of $^{15}\text{N}_2$ from ^{15}N -hydrazine in vivo

When hydrazine was injected intraperitoneally in single 1 mmole/kg doses, approximately 15% was converted to nitrogen gas within the first 30 minutes, followed by a much slower conversion over at least the subsequent 24 hours (Fig. 3 and Table 4, lines 1-6). The possibility that saturation of some reaction system slowed the conversion was examined by comparing $^{15}\text{N}_2$ production following i.p. treatment with doses of 0.5, 1.0 and 1.5 mmoles ^{15}N -hydrazine/kg. Recoveries were $16.0 \pm 3.26\%$, $16.8 \pm 1.53\%$ and $17.9 \pm 0.99\%$, respectively, indicating a slight, but nonsignificant increase associated with increased dose. An experiment in which two 1 mmole/kg doses were administered two hours apart provided recoveries slightly higher than should be expected for a single

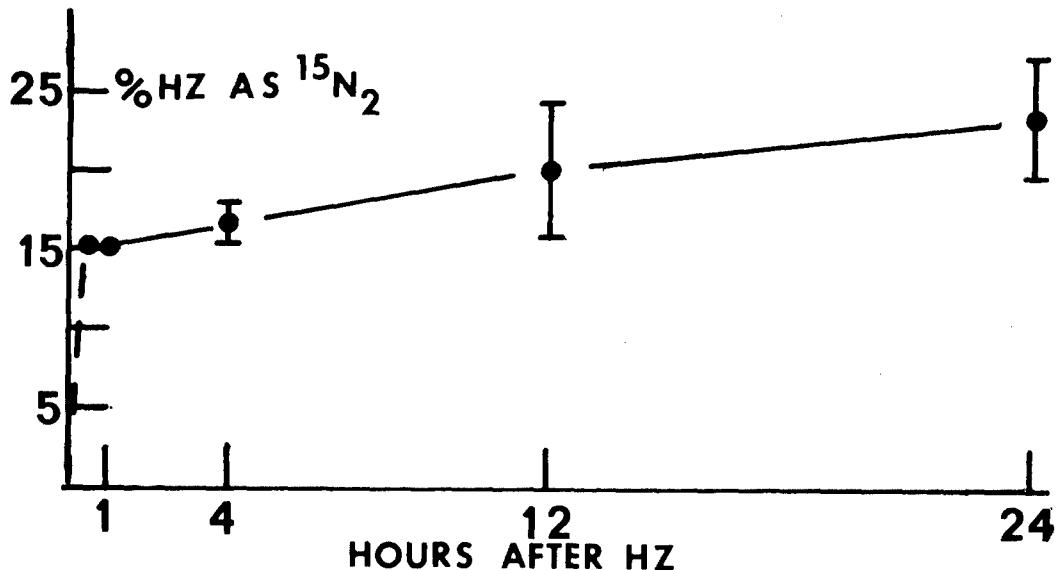


Figure 3. Conversion of ^{15}N -hydrazine to respiratory $^{15}\text{N}_2$ following a single 1 mmole/kg dose, ip. ($N=3$ at each time point).

2 mmole/kg dose, on the basis of the above dose-response information. Comparison of routes of administration indicated that over a 4 hour collection period the highest conversion arose from intravenous hydrazine, and the lowest following subcutaneous administration (Table 4, lines 4, 10, 12).

An attempt was made to observe the first few minutes of the course of the reaction by using intravenous administration of hydrazine in spite of the inherent variability associated with that route. Two samples obtained five minutes after injection were quite different (Table 4, line 8), but showed that significant reaction had taken place.

In view of the known reaction of hydrazine in oxygenated hemoglobin, two animals were maintained in 100% oxygen, resulting in somewhat decreased conversion (Table 4, line 17). To assure that all free nitrogen produced was adequately mobilized at termination, two animals were subjected to vacuum for 30 minutes after euthanasia but no significant increase in collection resulted (Table 4, line 18).

Formation of Ammonia

No $^{15}\text{N}_2$ has been detected in urinary ammonia of rats administered a single 1 mmole/kg dose of ^{15}N -hydrazine.

TABLE 4
FORMATION OF $^{15}\text{N}_2$ FROM ^{15}N -HYDRAZINE

Summary of effects of collection time following administration,
route of administration, dose and oxygen atmosphere.

<u>Line</u>	<u>Compare with Line #</u>	<u>Dose mmole /kg</u>	<u>Rt.</u>	<u>Coll. Time, Hour</u>	<u>N</u>	<u>$\bar{\mu} \pm s$</u>	
TIME COURSE OF $^{15}\text{N}_2$ PRODUCTION AFTER SINGLE DOSE OF ^{15}N -HYDRAZINE							
1		1	IP	0	2	$0.615 \pm .304$	A
2		1	IP	0.5	3	15.4 ± 3.18	
3		1	IP	1	3	15.3 ± 2.02	
4		1	IP	4	3	16.8 ± 1.53	
5		1	IP	12	2	20.2 ± 4.24	
6		1	IP	24	3	23.3 ± 3.76	
EFFECT OF INTRAVENOUS ADMINISTRATION							
7	1	1	IV	0	2	0.84 ± 0.45	A
8		1	IV	5 Min.	2	6.0 ± 3.44	
9	3	1	IV	1	3	14.4 ± 2.57	
10	4	1	IV	4	3	18.9 ± 5.30	
EFFECT OF SUBCUTANEOUS ADMINISTRATION							
11	3	1	SC	1	3	12.6 ± 1.64	
12	4	1	SC	4	3	14.0 ± 1.5	
EFFECT OF DOSAGE							
13		0.5	IP	4	4	16.0 ± 3.26	
14		1.0	IP	4	3	16.8 ± 1.53	B
15		1.5	IP	4	3	17.9 ± 0.99	
16		2.0	IP	4	2	20.8 ± 1.98	C
EFFECT OF 100% O ₂ ATMOSPHERE							
17	4	1.0	IP	4	2	12.0 ± 0.5	
COLLECTION UNDER 30 MIN VACUUM TO ASSURE RELEASE OF ENTRAINED $^{15}\text{N}_2$							
18	4	1.0	IP	4	2	17.4 ± 0.28	

A Animal euthanized at time of hydrazine injection.

B Line 4 repeated

C Two 1 mmole/kg doses two hours apart.

Reaction of Hydrazine with Hemoglobin and Other Blood Constituents

We are seeking an in vitro model that may be useful in explaining the mechanism of N_2 formation from hydrazine, and the nature of any other factors that may influence the reaction rate. Early studies with whole blood and blood constituents suggest that such a system may be a model for the sequence observed in vivo.

Whole blood converted ^{15}N -hydrazine to $^{15}N_2$ in vitro to a greater extent than did intact animals. Conversion under an atmosphere of 100% O_2 was not appreciably different from that in 20% oxygen, and both were quite variable. The time course of the reaction under 100% oxygen, based on the available data, shows a relatively constant rate of conversion for three hours, reaching a total of about 35% (Fig. 4). Whole blood incubated under 100% SF_6 converted $9.9 \pm 2.32\%$ ($N=5$) of ^{15}N -hydrazine to $^{15}N_2$ in three hours. The oxygenated system contained approximately 0.16 meq hemoglobin subunit and 0.115 mmole (15 mg) hydrazine sulfate in 22 ml; 0.230 mmole hydrazine was added to the deoxygenated series.

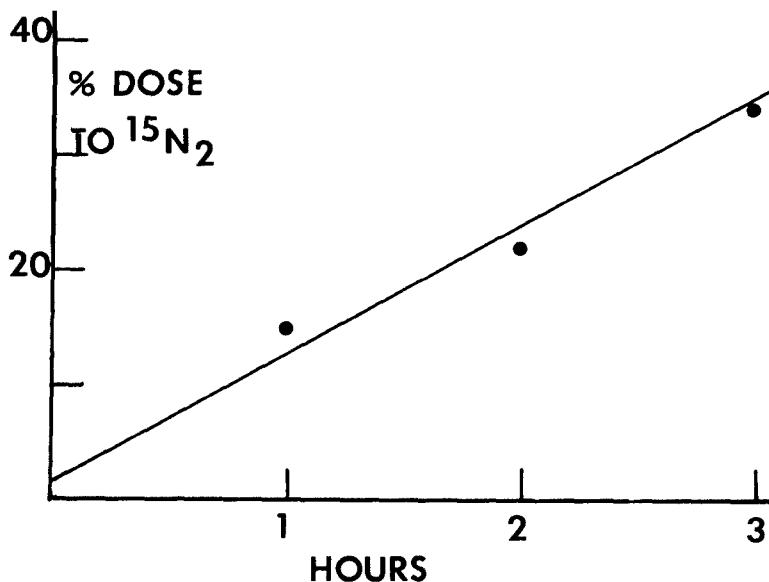


Figure 4. Conversion of ^{15}N -hydrazine to $^{15}N_2$ by whole blood, in vitro (0.16 meq hemoglobin subunit, 0.115 mmole hydrazine/22 ml blood, 100 O_2).

Solutions of hemoglobin under 100% oxygen and in the absence of other protein or cell membranes react with unlabeled hydrazine readily. The stoichiometry suggests that hemoglobin is either catalytic or serves as a replenishing oxygen carrier in the system. However, the kinetics of the reaction have not yet been fully studied.

Early experiments also show that bovine serum albumin protects hydrazine from reaction with hemoglobin and that the extent of reaction is related to the amount of albumin added. Surprisingly, rat plasma, without modification, did not confer such protection, and the reaction proceeded at the same rate as in buffer with no protein. Such factors as previously occupied albumin binding sites, influence of anticoagulants, and the role of the red cell membrane, either as a barrier or as a binding surface, are being examined.

Concentration of Hydrazine and Its Metabolite(s) in Blood Following Hydrazine Administration

During the first hour after a single ip injection of 1.0 mmole hydrazine /kg, blood hydrazine concentration declined rapidly, probably reflecting distribution and early metabolic activity. There appeared to be two separate subsequent phases between hours 1 and 6 and 6 and 24, associated with movement from tissues, excretion and slower metabolism. The half times of disappearance increased substantially for each successive phase. Data available are not sufficient to construct an adequate mathematical representation. At 24 hours, concentration of hydrazine and its metabolite was about 10% of the hydrazine concentration 15 minutes after administration (Fig. 5).

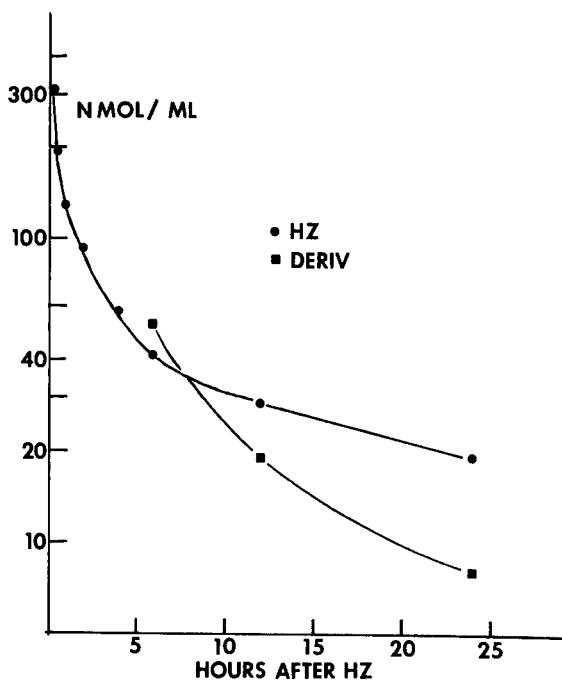


Figure 5. Concentration of hydrazine and a derivative(s) in blood after 1 mmole hydrazine /kg, single dose, ip. ($N = 6$ at 6 hours, 3 at all other points).

Continuous infusion of hydrazine over 6 - 12 hours indicates that at lower dose rates a steady state blood hydrazine concentration should be expected, proportional to input rate. Figure 6 shows data collected between hours 5 and 12 of infusion. Such regulation occurred very infrequently at dose rates in excess of 0.167 mmole/kg/hr.

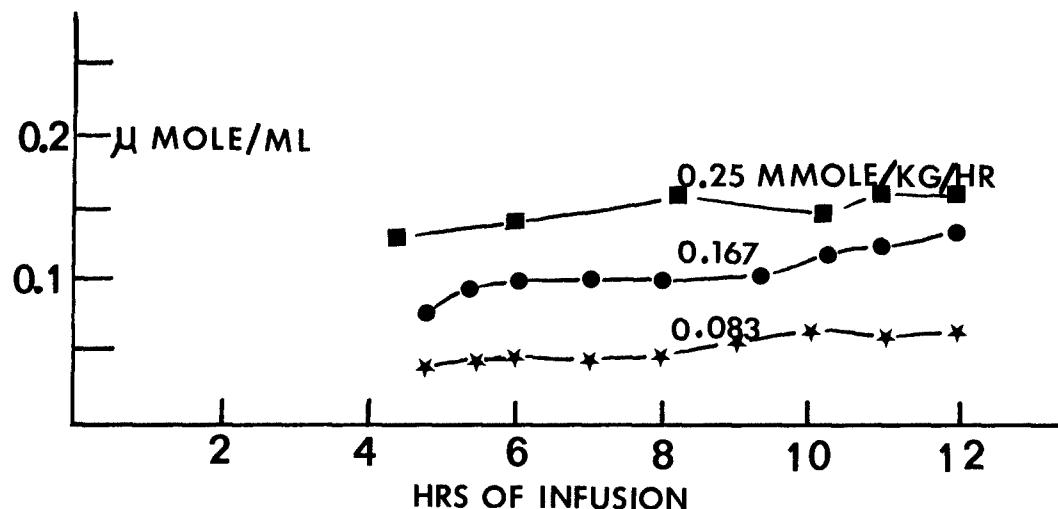


Figure 6. Concentration of hydrazine in blood during continuous sc infusion at indicated dose rates.

An attempt was made to identify a range of blood hydrazine concentrations that might be associated with lethal effect, but no clearcut relationship was found. Death has occurred at blood concentrations ranging from 0.3 - 1.05 μ mole hydrazine/ml. A number of continuously infused animals have been sampled over the time sequence immediately prior to death, in experiments intended to obtain other information. In most such cases, the blood hydrazine levels tended toward a slowly rising or constant concentration but after 8-10 hours the hydrazine level rose abruptly just prior to death.

Urinary Excretion of Hydrazine

The persistence of hydrazine in blood was paralleled by an extended period of excretion in urine. Rate of excretion of hydrazine and its metabolite(s) was in excess of 5% of the dose per hour immediately after administration, about 3% at 6 hours, 1% at 24 hours, and 0.25% at 48 hours (Figure 7). The excretion rate of the hydrazine metabolite was relatively constant through the first 6 hours after administration, and was not characterized by an initial high output rate. Cumulative excretion over 48 hours totaled about 52% (Figure 8), of which slightly more than half was hydrazine.

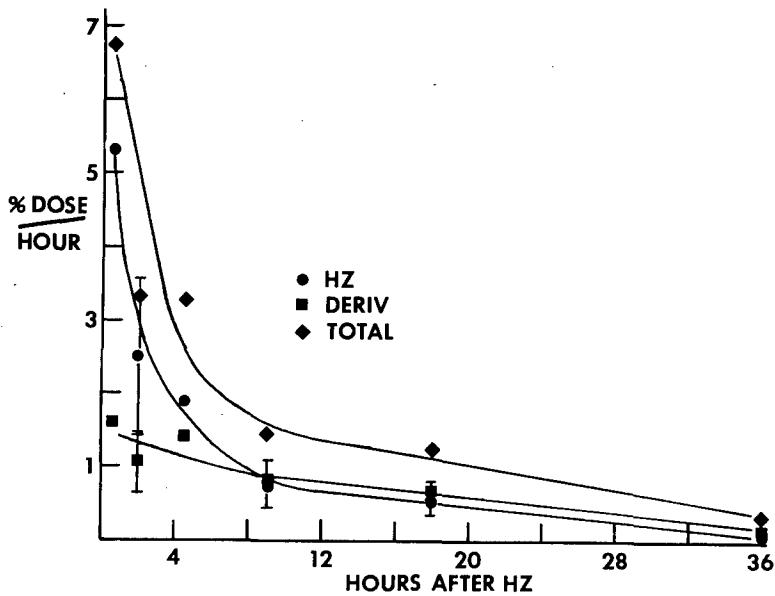


Figure 7. Urinary excretion rate of hydrazine and a hydrolyzable derivative(s) following a single 1 mmole/kg dose, ip. ($N = 4-10$ animals/point. Each point located in middle of collection period).

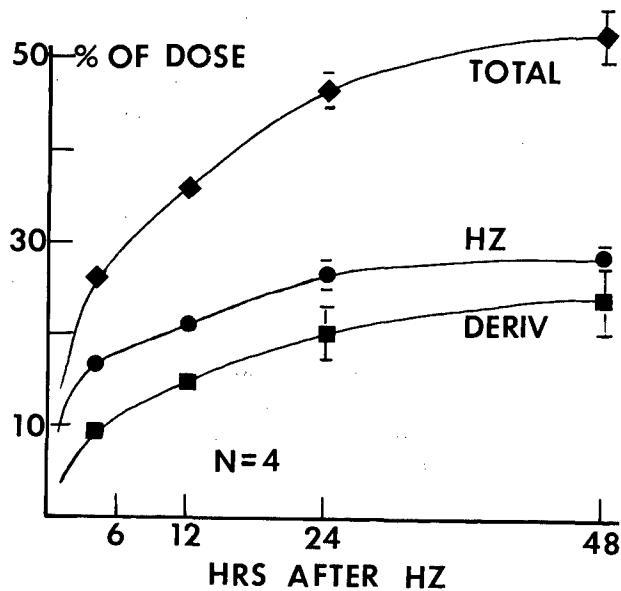


Figure 8. Cumulative excretion of hydrazine and a hydrolyzable derivative over 48 hours following a single 1 mmole/kg dose ip.

DISCUSSION

The time course of conversion of ^{15}N -hydrazine to $^{15}\text{N}_2$ presents an interesting pattern. The reaction is initially very rapid and is quite slow thereafter. There is some correspondence between the decline of nitrogen production and the decline in blood hydrazine. However, during the period from 1 to 3 hours, when nitrogen production had slowed sharply, blood hydrazine was higher than would be expected if a direct relation with nitrogen production exists. As more data are assembled, this relationship should become clearer. The kinetics of the conversion are clearly not a function of saturation of either a reaction system or of a sink for hydrazine, because the change in output relative to dose is negligible.

We speculate that the reaction is catalyzed by hemoglobin. We find that in whole blood 50% of added ^{15}N -hydrazine was converted to $^{15}\text{N}_2$ in three hours, and in a cell-free hemoglobin solution conversion exceeded 80%. In preliminary experiments, addition of albumin to hemoglobin solutions decreased loss of hydrazine from solution, presumably through binding of hydrazine. Fresh serum had no effect.

We are extremely interested in the nature of this system, because it seems reasonable that the very rapid metabolic activity might be exploited in some way for reversal of acute hydrazine intoxication. The general problem is to find a way of increasing the fraction of circulating hydrazine that is accessible to the reaction.

A more complete inventory of administered hydrazine has been achieved than past studies have accomplished. About 23% of administered hydrazine emerged as nitrogen gas at 24 hours, and a recovery of about 25% can be assumed by extrapolation to 48 hours. Cumulative recovery of hydrazine and its derivatives in urine was slightly in excess of 50%, totalling, with $^{15}\text{N}_2$, more than 75%. Both $^{15}\text{N}_2$ production and urinary excretion were very nearly complete by 48 hours, suggesting that either an unusually large fraction of hydrazine was tissue bound, or that an undetected metabolite exists.

There is an interest in the expected behavior of hydrazine at steady, low level intake, as a simulation of possible work place exposure. Animals continually infused with hydrazine at relatively low rates are able to maintain a constant blood hydrazine concentration for at least 12 hours of infusion. The steady state may be established as early as two hours after beginning of infusion but occasionally requires 4-5 hours.

The absence of ^{15}N in urinary ammonia is interesting, in view of the potential for ammonia formation from hydrazine in non-biological reactions, and in view of the suggestion by Procellati and Preziosi (1954) that intact rabbits and isolated rat tissues could enzymatically make that conversion.

We have begun work on emission spectroscopy techniques which should be considerably more sensitive for measuring ^{15}N in tissues, whether as hydrazine or as a product, and we are hopeful that these questions can be resolved in the near future.

REFERENCES

Buckmaster, G.A. (1913). The behavior of blood with hydrazine hydrate. Jour. Physiol. (Proceedings) 46:XVIII.

Buckmaster, G.A. (1914). Action of hydrazine hydrate on methemoglobin. Jour. Physiol. (Proceedings) 48:XXV.

Conway, E.J. (1957). Microdiffusion and volumetric error. 4th Ed. Crosby and Lockwood, London.

Dambrauskas, R. and H.H. Cornish (1964). The distribution, metabolism, and excretion of hydrazine in rat and mouse. Toxicol. Appl. Pharmacol. 6:653.

Dufour, A.P., R.A. Knight and H.W. Harris (1964). Genetics of isoniazid metabolism in Caucasian, Negro, and Japanese populations. Science 145:391.

Evans, D.A.P., K.A. Manley and V.A. McKusick (1960). Genetic control of isoniazid metabolism in man. Br. Med. Jour. II:485.

Liu, Y.-Y., S. Schmeltz and D. Hoffmann (1974). Chemical studies on tobacco smoke. Quantitative analysis of hydrazine in tobacco and cigarette smoke. Anal. Chem. 46:885.

McKennis, H. and J.H. Weatherby (1956). Blood ammonia following administration of various hydrazino compounds. Fed. Proc. 15:458.

McKennis, H., A.S. Yard, J.H. Weatherby and J.A. Hagy (1959). Acetylation of hydrazine and the formation of 1,2,-diacetylhydrazine in vivo. J. Pharm. Exptl. Therap. 126:109.

Porter, L.K. and W.A. O'Deen (1977). Apparatus for preparing nitrogen from ammonium chloride for nitrogen-15 determinations. Anal. Chem. 49:514.

Porcellati, G. and P. Preziosi (1954). Biological transformation of iso-nicotinoyl hydrazine. Enzymologia 17:47.

Reynolds, B.A. and A.A. Thomas (1965). A colorimetric method for the determination of hydrazine and monomethylhydrazine in blood. Am. Ind. Hygiene Assoc. Jour. 26:527.

Ross, P.J. and A.E. Martin (1970). A rapid procedure for preparing gas samples for nitrogen-15 determination. Analyst 95:817.

Turner, R.A. (1947). Reactions of semicarbazones. J. Amer. Chem. Soc. 69:875.

Wellman, C.R., J.R. Ward and L.P. Kuhn (1976). Kinetics of the oxidation of hydrazine by hydrogen peroxide, catalyzed by hydrated copper (II). Amer. Chem. Soc. 98:1683.